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Cilostazol Stimulates Revascularisation in Response to Ischaemia via an eNOS-Dependent Mechanism☆

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ABSTRACT

Objectives: Cilostazol is known to be a selective inhibitor of phosphodiesterase 3 and is generally used to treat intermittent claudication caused by peripheral arterial disease. However, there is little information concerning the effect of cilostazol on angiogenesis. Here, we investigated whether cilostazol modulates the angiogenic process *in vivo* employing a hindlimb model of ischaemia-induced angiogenesis.
Design: This was an experimental study.
Materials and methods: Wild-type (WT) mice were randomly divided into two groups and were treated with or without cilostazol. One week later, the mice were subjected to unilateral hindlimb ischaemia. Angiogenesis was determined by laser Doppler analysis and capillary density stained with CD31. The expression of endothelial nitric oxide synthase (eNOS) was assessed by immunoblotting.
Results: WT mice treated with cilostazol showed accelerated neo-vascularisation following hindlimb ischaemic surgery on post-operative day 14 based upon laser Doppler measurements of blood flow (cilostazol-treated group, 0.54 ± 0.13 vs. control group, 0.38 ± 0.11; $P<0.05$). The capillary density in the ischaemic hindlimb was also significantly greater in WT mice treated with cilostazol than in non-treated WT mice (cilostazol-treated group, 1.63 ± 0.10 vs. control group, 1.15 ± 0.12; $P<0.01$). Cilostazol stimulated an ischaemia-induced increase in the phosphorylation of eNOS in the ischaemic limbs. Administration of NOS inhibitor N-nitro-L-arginine methyl ester (L-NAME) abolished cilostazol-induced increase in limb perfusion.
Conclusions: Our observations indicate that cilostazol can promote neo-vascularisation in response to tissue ischaemia via an eNOS-dependent mechanism. Cilostazol could be useful for treatment of ischaemic limb diseases.

Introduction

A selective inhibitor of phosphodiesterase 3 (PDE3), cilostazol is widely used for the treatment of peripheral arterial disease (PAD).[1] Treatment with cilostazol improved exercise performance, walking ability and reduced the negative effects on quality of life associated with claudication, the major symptomatic manifestation of PAD.[2] Consistent with these clinical observations, several experimental studies have indicated that cilostazol has beneficial effects on the vascular wall, including improvement of endothelial function.[3] Cilostazol acts on platelets, vascular smooth muscle cells and endothelial cells through an elevation in cyclic adenosine monophosphate (cAMP) levels by a combination of the inhibition of intracellular PDE-3A and extracellular adenosine uptake.[4] These findings suggest that cilostazol exhibits anti-platelet aggregation and vasodilatation properties. However, the effects of cilostazol on vascular responses to tissue ischaemia have not been examined. In the present study, we investigated the effect of systemic administration of cilostazol on the ischaemia-induced neo-vascularisation process *in vivo* and examined the potential involvement of endothelial nitric oxide synthase (eNOS) signals in the regulation of cilostazol-regulated neo-vascularisation in ischaemic muscles.

Materials and Methods

An eNOS antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-eNOS (Ser-1177) was purchased from Cell Signaling Technology (Beverly, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Biogenesis Inc. N-Nitro-L-Arginine Methyl Ester (L-NAME) was purchased from Sigma Chemical Co (St. Louis,

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Animals and experimental protocol

Male wild-type (WT) mice with a C57BL/6J background at the age of 8–10 weeks were used in this study. The study protocol was approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. We used a mouse model of neo-vascularisation, in which the entire left femoral artery and vein were removed surgically as previously described.[5] The mice were randomly divided into two groups and were treated with or without cilostazol as food admixture at a concentration of 0.1% consistent with previous reports.[6,7] One week later, the mice were subjected to unilateral hindlimb ischaemia. In some experiments, NOS inhibitor L-NAME (20 mg kg^{-1} day^{-1}) dissolved in phosphate-buffered saline (PBS) or vehicle (PBS) was intra-peritoneally injected into the abdomen of WT mice 1 day before the operation until sacrifice.[8]

Laser Doppler blood flow analysis

We measured hindlimb blood flow using a laser Doppler blood flowmetry (LDBF; MoorLDI, Moor Instrument, Devon, UK), as previously described.[8,9] Before and on post-operative days 0, 3, 7 and 14, we performed LDBF analysis on the legs and feet. After scanning, stored images were analysed to quantify blood flow, and mean LDBF values of the ischaemic and non-ischaemic limbs were calculated. To avoid data variations due to ambient light and temperature, hindlimb blood flow was expressed as the ratio of the left (ischaemic) to right (non-ischaemic) hindlimb LDBF. The assessments were made in a blinded fashion.

Capillary density analysis

Capillary density in adductor muscle was analysed to obtain specific evidence of vascularity at the level of microcirculation. Tissue samples were obtained from the ischaemic thigh adductor skeletal muscles on post-operative day 14. Frozen tissue sections of 5-μm thickness were prepared from each sample. Capillary endothelial cells were identified by immunohistochemical staining with CD31 monoclonal antibody (BD Biosciences, Franklin Lakes, NJ, USA). Fifteen random microscopic fields from three different sections in each tissue block were examined for the presence of capillary endothelial cells, and capillary/muscle fibre ratio was expressed as the ratio of number of capillaries to the number of myofibres per high-power field (×400).[10]

Western blot analysis

Tissue samples obtained on post-operative day 5 were homogenised in lysis buffer containing 20 mM Tris–HCl (pH 8.0), 1% Noniet P-40, 150 mM NaCl, 0.5% deoxycholic acid, 1 mM sodium orthovanadate and protease inhibitor cocktail (Sigma Chemical Co, St, Louis, MO, USA). Protein content was determined by the Bradford method. The same amounts of protein (60 μg) were separated with denaturing sodium dodecyl sulphate (SDS) 10% polyacrylamide gels. The membranes were immune blotted with the primary antibodies at a 1:1000 dilution, followed by secondary antibody at a 1:5000 dilution. Bands were visualised using the ECL Western Blotting Detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). All the data were subjected to one-way analysis of variance (ANOVA) followed by Scheff's analysis. P values < 0.05 were considered to be statistically significant.

Results

Cilostazol promotes neo-vascularisation in response to tissue ischaemia

To assess the effect of cilostazol on the neo-vascularisation process in response to ischaemia, WT mice treated with or without cilostazol were subjected to unilateral hindlimb ischaemia. All mice survived after surgery and appeared healthy during the follow-up period. Fig. 1(A) shows representative LDBF images of hindlimb blood flow before surgery and at different time points after surgery. Blood flow recovery in the ischaemic hindlimb appeared to be accelerated in the cilostazol-treated WT mice compared with the untreated (control) mice. Quantitative analysis of hindlimb perfusion showed that treatment with cilostazol significantly increased the limb flow of ischaemic muscle in WT mice on post-operative day 14 (cilostazol-treated group, 0.54 ± 0.13 vs. control group, 0.38 ± 0.11; $P < 0.05$) (Fig. 1(B)).

To assess the extent of neo-vascularisation at the microcirculatory level, we measured capillary density in a histological section harvested from the ischaemic muscle. Fig. 2(A) shows

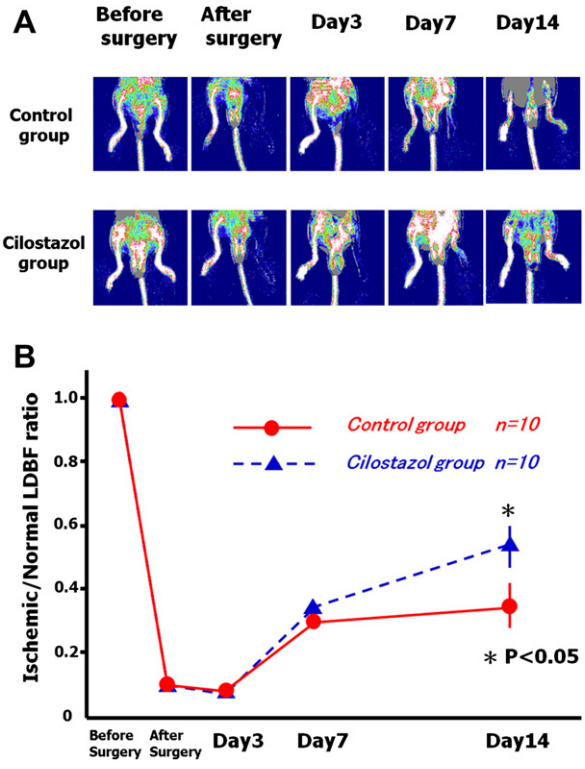

Figure 1. Improved angiogenic response in the ischemic hindlimb of cilostazol-treated WT mice. (A) Representative images of LDBF for WT mice treated with or without cilostazol before surgery and at different time points after surgery. A low perfusion signals (dark blue) was observed in the ischemic hindlimb of WT mice, whereas a high perfusion signals (white to red) was detected in WT mice treated with cilostazol on post-operative day 3, 7 and 14. (B) Quantitative analysis of ischemic/normal LDBF ratio in WT mice ($n = 10$ in each groups) treated with or without cilostazol (*$p < 0.05$ vs. control).

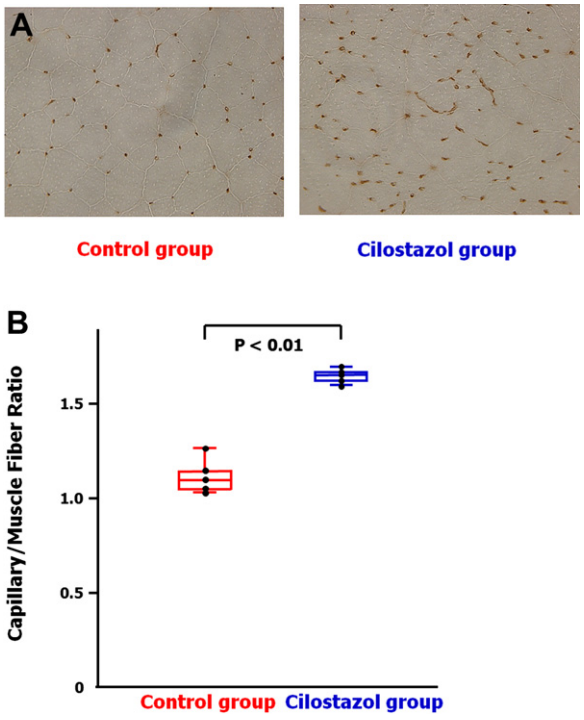

Figure 2. Increased capillary density in ischemic cilostazol-treated WT mice. (A) Immunostaining of ischemic tissues with anti-CD31 monoclonal antibody (brown) on post-operative day 14 (×400). (B) Quantitative analysis of capillary density in WT mice ($n = 5$ in each groups) treated with or without cilostazol.

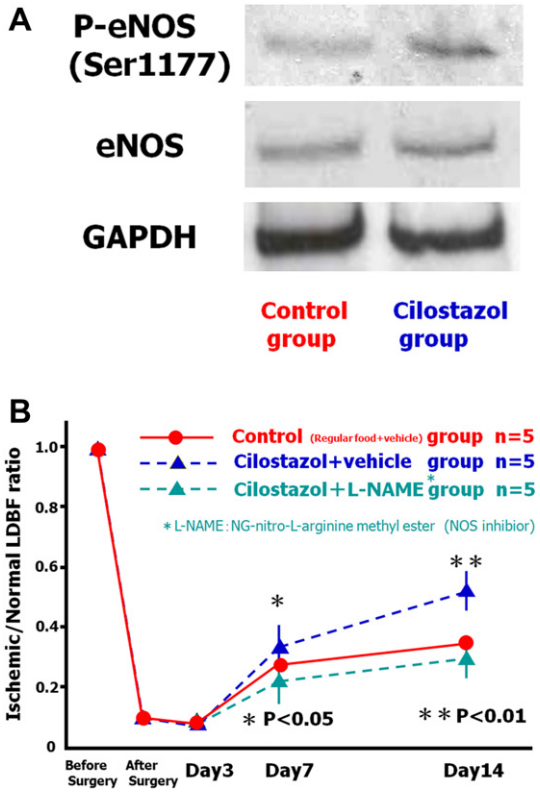

Figure 3. eNOS pathway is required for cilostazol-induced neovascularization. Western immunoblots with the indicated antibodies were performed on the ischemic adductor muscle of WT mice treated with or without cilostazol at 5 days after surgery. (A) The representative immunoblots and quantitative analysis of relative changes in phosphorylated eNOS. Phosphorylation of eNOS were normalized to the GAPDH signal and expressed as percentage of the signal intensity of untreated WT mice ($n = 3$). (B) Quantitative analysis of ischemic/nonischemic LDBF ratio in non-treated or cilostazol-treated WT mice in the presence of L-NAME, or vehicle before surgery and at different time points after surgery ($n = 5$ in each groups). (*$p < 0.05$ **$p < 0.01$ vs. control).

representative photomicrographs of muscle tissue stained with the endothelial cell marker CD31. Quantitative analysis of CD31-positive cells revealed that, on post-operative day 14, the capillary density in the ischaemic hindlimb was significantly greater in WT mice treated with cilostazol than in control mice (cilostazol-treated group, 1.63 ± 0.10 vs. control group, 1.15 ± 0.12; $P-<-0.01$) (Fig. 2(B)).

eNOS activation is essential for cilostazol-induced neovascularisation.

eNOS plays an important role in neo-vascularisation following hindlimb ischaemia. To analyse the potential involvement of eNOS in cilostazol-induced revascularisation, expression and phosphorylation of eNOS in ischaemic adductor muscle at day 5 after surgery were assessed using Western blot analysis. The expression of total eNOS protein in ischaemic muscles did not differ between the cilostazol-treated WT mice and the untreated mice. However, phosphorylation of eNOS at Ser-1177 in ischaemic muscle was significantly greater in the cilostazol-treated WT mice than in the untreated mice (Fig. 3(A)). To further analyse the involvement of eNOS signalling in the enhancement of neo-vascularisation by cilostazol, we examined the effect of NOS inhibitor L-NAME on recovery of blood flow of ischaemic muscles in the WT mice receiving cilostazol. Treatment of the WT mice with L-NAME blocked the increased limb perfusion caused by cilostazol (Fig. 3(B)). Collectively, these data suggest that cilostazol-stimulated neo-vascularisation is attributed to eNOS activation.

Discussion

The present study demonstrates that systemic administration of cilostazol stimulates neo-vascularisation in response to ischaemia in a mouse model of vascular insufficiency. Treatment of the WT mice with cilostazol resulted in a more rapid recovery of limb perfusion and enhanced capillary density compared with the untreated mice, which was accompanied by increased levels of eNOS phosphorylation. The beneficial actions of cilostazol on neovascularisation were abrogated in the WT mice receiving NOS inhibitor.

In 1988, cilostazol was approved in Japan for the treatment of ulcerations, pain and coldness associated with PAD.[11] An increasing number of clinical trials reported that cilostazol therapy is associated with lower risk of limb amputations and improved ischaemic symptoms, such as intermittent claudication, in patients with PAD.[12–16] However, the precise mechanism of how cilostazol regulates these vascular effects is incompletely understood. As one of the possible mechanisms, it is known that cilostazol inhibits cAMP phosphodiesterase III, resulting in decreased phosphodiesterase activity and suppression of cAMP degradation.[17,18] In turn, the level of cAMP in platelets and blood vessels is increased, leading to inhibition of platelet aggregation and to vasodilatation, respectively.[19,20] In addition to these effects, our observations show that cilostazol exerts a pro-angiogenic effect in ischaemic tissue by acting directly on the vascular endothelium. Therefore, our findings could provide important basic data explaining the beneficial effects of cilostazol for the treatment of PAD.

The ability of cilostazol to increase eNOS activation is likely to have contributed to the stimulation of neo-vascularisation under our experimental conditions. It is well established that eNOS is beneficial for various types of vascular diseases.[21] It has been

shown that cilostazol yields endothelial protection mediated by inhibition of lipopolysaccharide-induced apoptosis and induced NO production by eNOS activation in endothelial cells.[22–24] Cilostazol-induced NO production by eNOS stimulates capillary-like tube formation in endothelial cells.[24] Cilostazol also protects mice subjected to cerebral ischaemia by improvement of regional cerebral blood flow after ischaemia/reperfusion, in association with eNOS activity *in vivo*.[25] In the present study, cilostazol increased the activating phosphorylation of eNOS in ischaemic muscles, and the ability of cilostazol to enhance revascularisation was abrogated in the WT mice receiving NOS inhibitor. Collectively, these observations suggest that eNOS acts as a key mediator of the vascular protective actions of cilostazol.

In the present study, cilostazol promoted angiogenic repair in ischaemic limbs through its ability to increase eNOS activation. Thus, the pro-angiogenic effect of cilostazol could represent a common mechanism in the vascular protection by this reagent. Cilostazol could be beneficial for treatment of ischaemic heart and limb diseases.

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Conflict of interest

None.

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